after decarboxylation does not change the state of activation of the enzyme.

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Registry No. Pyruvate decarboxylase, 9001-04-1; pyruvate, 127-17-3; 3-fluoropyruvate, 433-48-7.

Effect of Heme Orientation on the Reduction Potential of Cytochrome b_5

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Abstract: Bovine erythrocyte cytochrome b_5, a soluble protein, and bovine microsomal cytochrome b_5, solubilized by trypsin cleavage, were isolated and purified, and their proton NMR spectra were compared. These two proteins had identical chemical shifts of the heme resonances and the same ratio of major to minor heme orientation. Thus, spectroelectrochemical titrations were carried out on the trypsin-cleaved microsomal protein. In its equilibrium ratio of 9:1 major to minor heme orientation, cytochrome b_5 gave a reduction potential of -1.9 ± 1.6 mV vs SHE at $\mu = 0.13$ M, pH 7.0, and $T = 24$ °C, utilizing three electrochemical mediators, 0.2 mM $Ru(NH_3)_6Cl_3$, 0.2 mM $K_3Fe(CN)_6$ and 1 mM methyl viologen. Spectroelectrochemical titrations of the apoprotein freshly reconstituted with hemin, in which the major:minor ratio was close to 1:1, yielded a reduction potential of -10.0 ± 1.7 mV vs SHE under the conditions listed above. The potentials of two samples of reconstituted cytochrome b_5 that had been allowed to equilibrate for 3 days were both -1.8 mV, indicating a return to the equilibrium ratio of major to minor forms. NMR quantitation of the time course of heme rotation in H_2O under the electrochemical conditions has led to an estimate of major:minor ratio of 60:40 at the midpoint of the titration and a half-life for heme reorientation of 12 ± 1 h. These data lead to calculated reduction potentials of $+0.8$ and -26.2 mV, respectively, for pure major and minor heme orientations. Although this difference is probably not large enough to be physiologically significant, the fact that the difference exists suggests that a detailed correlation between structure and reduction potential is very important for understanding the reduction potentials of heme proteins in general.

Cytochrome b_5 is a heme protein that exists in soluble form in erythrocytes and in membrane-bound form in liver microsomes. Erythrocyte cytochrome b_5 has been shown to mediate the reduction of methemoglobin by NADH-cytochrome b_5 reductase in normal red cells.[1-3] The presence of cytochrome b_5 in erythrocytes stimulates methemoglobin reduction by NADH-cytochrome b_5 reductase by as much as 77-fold.[1] The protein is thus important in reducing the ca. 3% of hemoglobin that is oxidized to the met form each day in normal humans.[4] Low levels of erythrocyte cytochrome b_5 may be associated with some forms of methemoglobinemia.[1]

Microsomal cytochrome b_5 is a membrane-bound amphiphatic redox protein that functions as a component of a microsomal electron-transfer chain in endoplasmic reticulum membranes. It has also been shown to be bound to outer mitochondrial membranes[5] and in plasma membranes from intestinal microvilli and erythrocytes[6] as well as in the mitochondrial intramembrane space.[7] There is evidence that it participates in the microsomal stearyl-CoA desaturation reaction.[8] It is also known to interact with cytochrome P450 as an alternate electron donor and to act as a respiratory carrier during hepatic microsomal mixed-function oxidation reactions.[9] It is a two-domain protein, with a hydrophilic, globular, heme-containing domain that is anchored to the membrane by a hydrophobic tail.[10] The membrane-bound protein can be solubilized by detergents or by treatment with trypsin, which cleaves the hydrophobic tail, as well as the first three amino acids of the N-terminus.[11,12]

The protein sequence of the two individual types of bovine erythrocyte cytochrome b_5 is the same as the first 95 or 97 (types I and II, respectively) amino acids of bovine microsomal b_5, indicating that the two proteins may be specified by the same gene. It has been suggested that the soluble erythrocyte protein is derived from the microsomal protein by proteolysis during erythroid maturation.[13] Trypsin cleavage of microsomal cytochrome b_5 produces a protein that consists of residues 3-86 of the original membrane-bound protein.[11,12] It thus differs from the type II erythrocyte protein in having 3 less amino acids at the N-terminal end and 11 less at the C-terminal end.

Trypsin-cleaved cytochrome b_5 has been the subject of several NMR spectroscopic investigations.[14-18] Ferricytochrome b_5

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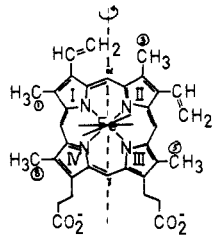

Figure 1. Diagram of the heme group, showing the approximate orientation of the axial histidine ligands in the major form of cytochrome b_5 and indicating the rotation axis that results in heme disorder in this and other *b*-type heme proteins.

contains low-spin Fe(III), which is paramagnetic. Thus, most of the heme proton resonances are shifted well outside the diamagnetic region of the NMR spectrum. Careful study of these resonances, including the reconstitution of apocytochrome b_5 with modified hemes,[16] has allowed assignment of the majority of the heme resonances and has shown that the native protein exists in two forms, a "major" and a "minor", in the approximate equilibrium ratio of 9:1, which are interconvertible on a slow time scale following reconstitution of the apoprotein with hemin.[16] It was shown by use of modified hemes that the two forms resulted from "heme disorder", or rotation of the heme moiety about the α,γ meso axis,[16] as shown in Figure 1. Such rotation switches the positions of the methyl and vinyl substituents of pyrrole rings I and II, placing these substituents in different environments with respect to the protein side chains that press against the heme in its pocket. It also has the effect of changing the orientation of the two coordinated histidine imidazole planes from lying approximately above and below pyrrole rings II and IV (major) to above and below rings I and III (minor). The half-life for heme rotation in ferricytochrome b_5 in D_2O is 3.6 h at pH 8[19] and 21.5 h at pH 7 and 25 °C.[20] However, ferrocytochrome b_5, the form in which the erythrocyte protein is maintained by NADH-cytochrome b_5 reductase, has a much longer half-life for heme rotation, of the order of 100 times that of the oxidized protein.[20]

Similar NMR studies showed heme disorder in myoglobins and hemoglobins,[21-22] and in the case of myoglobin, investigation of oxygen binding to the protein immediately following reconstitution with hemin showed increased affinity of the protein for molecular oxygen.[23] The equilibrium constant for binding of O_2 to the minor form was more than a factor of 10 greater than that for the major form.[23] These results, coupled with the additional finding that the heme orientational ratio in freshly killed yellowfin tuna muscle was not the equilibrium ratio, but rather was relatively rich in the minor isomer,[24] suggested a time-dependent capability of muscle tissue to store oxygen. Light and co-workers[25] recently reinvestigated the equilibrium and kinetic parameters for O_2 and CO binding to freshly reconstituted and native sperm whale myoglobin. They concluded that the orientation of the heme group has no effect on the physiological properties of myoglobin.

Irrespective of the role of heme orientation on the properties of myoglobin, we wondered whether one possible reason for the decreasing effectiveness of the methemoglobin reductase system of red blood cells as a function of time[26] might be that the reduction potential of cytochrome b_5 was dependent on the degree of heme disorder, that is, on the ratio of major to minor forms. Implied in this hypothesis is the fact that the rate of heme reorientation is slow under physiological conditions. That this is the case, at least in D_2O, has been shown recently by LaMar and McLachlan,[20] as mentioned above.

In order to test the above hypothesis, we first isolated bovine erythrocyte and trypsin-cleaved bovine microsomal cytochromes b_5 and compared their NMR spectra. Once it became clear that the heme environment and equilibrium orientational ratio were the same in these two proteins, we then concentrated our electrochemical investigations of cytochrome b_5 on the trypsin-cleaved microsomal protein, because of the comparative ease of isolation of this form of cytochrome b_5.

Experimental Section

Materials. Chemicals were obtained from Spectrum or Sigma, unless otherwise indicated. Potassium ferricyanide and ruthenium hexammine trichloride were obtained from Alfa. All chemicals were used as received unless otherwise indicated. Fresh bovine blood was obtained from McDermott's Beef Co., Berkeley, CA; fresh bovine liver was obtained from Hohner's Meat Co., San Leandro, CA. DEAE cellulose (Sigma), DE52 (Whatman), Bio-Gel P-60 100–200 mesh (Bio-Rad), DEAE Sephadex A50–120 (Sigma or Pharmacia), DEAE Trisacryl (LKB), and G75-40 Sephadex (superfine; sigma) resins were prepared and utilized according to established procedures. Dialysis tubing was obtained from VWR Scientific and from Spectrapore. All water used was either glass distilled or deionized by use of a Super Millipore filter system.

Isolation of Bovine Erythrocyte Cytochrome b_5. Cytochrome b_5 was isolated from bovine erythrocytes by elaboration of the method of Hultquist et al.[13] The detailed procedures are given in ref 31. The final yield, from a total of 60 L of bovine blood, was 8.8 mg, with an R value of 5.9.

Isolation of Trypsin-Cleaved Bovine Microsomal Cytochrome b_5. Cytochrome b_5 was trypsin solublized, isolated, and purified by the method of Reid and Mauk.[27] From a liver weighing between 15 and 18 lb, approximately 120 to 150 mg of cytochrome b_5 (R = 6.0 for the central fractions, 3.8 for peripheral fractions) was obtained following two successive DE52 columns and a G75 Sephadex column, as described by Reid and Mauk.[27]

Heme Reconstitution. Apocytochrome b_5 was prepared by the acid acetone method of Strittmatter[28] and by the butanone method of Reid.[29] Both methods were acceptable for preparing apocytochrome b_5, but the butanone method was preferred because better yields were obtained, larger quantities of the protein could be prepared per separation, and it was less time consuming than the acid acetone method. After the apoprotein was concentrated, it was reconstituted by first adjusting the pH to 7.5 and then titrating an aliquot of the apoprotein with a solution containing 1.6 mg of hemin in 1.0 mL of 0.1 M KOH (prepared under vigorous stirring with a vortex mixer for 5 min) while monitoring the Soret band of cytochrome b_5 at 412 nm. The absorbance was plotted vs the volume of hemin solution; the amount of hemin required to fully form the holoprotein was determined from the break in the curve. The calculated volume of hemin solution necessary to reconstitute the desired quantity of apocytochrome b_5 was then added, and the pH of the resulting solution was immediately adjusted to 7.2. In some cases, the solution was then quickly filtered through a 0.8 × 5 cm Sephadex G25 fine column. In most cases, however, only 95% of the calculated amount of hemin was added, followed by immediate pH adjustment to 7.2. This ensured that there would be no free hemin in the solutions used for electrochemistry, since it was found that free hemin adsorbed on the electrodes and also shifted the midpoint potential obtained from the Nernst plot. As soon as the reconstitution was completed, the three mediators and the $NaClO_4$ were added. The final solution pH was 7.0.

NMR Spectroscopy. Samples of cytochrome b_5 utilized for NMR spectroscopy were prepared in either H_2O or D_2O. Transfer of the cytochrome b_5 samples from H_2O to mainly D_2O, when desired, was accomplished by means of 1:100 dilution of the protein with D_2O, followed by concentration utilizing an Amicon ultrafiltration cell equipped with a 25-mm YM-2 membrane. In several cases the apoprotein was lyophilized and then dissolved in D_2O, followed by reconstitution with hemin as described above. All resulting NMR samples had a volume of 0.5 mL and a cytochrome b_5 concentration of ~1 mM. Adjustments of

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pH were accomplished by addition of small amounts of 0.1 M NaOD or 0.1 M DCl, as necessary, utilizing a Radiometer TTT2 titrator unit equipped with a glass, calomel combination microelectrode. pH values are not corrected for the deuterium isotope effect. Samples in H_2O utilized for the measurement of the rate of heme rotation under the conditions used for spectroelectrochemical titrations were prepared as described below, except that the reconstituted apocytochrome b_5 and heme concentrations were ~1 mM. The temperature of the samples was 24 °C throughout the NMR experiments.

NMR spectra were recorded on the Nicolet NT-360 NMR spectrometer at the University of California, Davis, or on the General Electric GN-300 NMR spectrometer at San Francisco State, each utilizing quadrature detection. The soft pulse water proton saturation experiment SPLAPO (U.C. Davis NMR Facility) or the 1331 pulse sequence (SF-SU) was utilized to decrease the intensity of the water signal. Approximately 1000 transients were collected, with a 1-s delay between pulses. A 5-Hz apodization was applied to the fid before transformation. Spectra were referenced to HDO at 4.76 ppm. Integration of the peaks at 18.5 ppm (6-α-CH_2 minor) and 19.7 ppm (7-α-CH_2 major) was utilized in order to determine the ratio of major to minor heme orientations and the rate of heme reorientation when the 1331 pulse sequence was used, since the proximity of these two resonances ensures very similar transmitter power. Kinetic data were analyzed in terms of a reversible first-order reaction.[30] If A is the minor orientation and B is the major orientation of cytochrome b_5, the reaction can be represented by

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \tag{1}$$

If A and B are the concentrations of minor and major heme orientations at time zero, then the change in concentration of A (C_A) is

$$-dC_A/dt = k_1C_A - k_{-1}C_B \tag{2}$$

The change in concentration of B (C_B) is

$$dC_B/dt = k_1C_A - k_{-1}C_B \tag{3}$$

The concentration of minor and major species at time t can then be represented by $(A - x)$ and $(B + x)$. Then

$$dx/dt = k_1(A - x) - k_{-1}(B + x) \tag{4}$$

If X_e is the value of x at equilibrium and X_0 is the value of x at time 0, then

$$(k_1 + k_{-1})t = \ln [(X_e - X_0)/(X_e - x)] \tag{5}$$

A plot of the term $\ln [(X_e - X_0)/(X_e - x)]$ vs time should be linear with a slope of $(k_1 + k_{-1})$. The equilibrium constant $K_{eq} = k_1/k_{-1} = 9$, as measured previously[22] and in this work.

Spectroelectrochemistry. Preliminary bulk electrochemical titrations of cytochrome b_5 were carried out in the laboratory of Dr. George Wilson of the University of Arizona.[31] During these titrations, problems were encountered with the extreme ease of autoxidation of this protein in the bulk electrochemical cell system[32] and with the considerable amount of time (6 h) required to complete a titration, due to the volume of the solution and the fact that a conventional recording spectrophotometer was being utilized. Because we wished to investigate solutions containing nonequilibrium ratios of major to minor heme orientations of cytochrome b_5, which would interconvert with a half-life of 1 day or somewhat less, a more rapid technique was desired. Therefore, thin-cell spectroelectrochemical techniques were utilized, and all UV/visible spectroscopic measurements reported herein were made on a Hewlett-Packard 8451A diode array spectrophotometer equipped with a Hewlett-Packard 7470A graphics plotter. Spectra were stored on 120-mm disks. A Princeton Applied Research potentiostat, Model 173, was used for these spectroelectrochemical titrations.

The anaerobic spectroelectrochemical thin cells are modified forms of a design by Hawkridge and Kuwana[33] and Heineman and co-workers[34] and were designed according to a prototype cell provided by Balfe.[35,36] The cell bodies were constructed of Lucite and had a gold minigrid optically transparent thin-layer electrode (OTTLE) (200 wires per inch, 70% transmittance, Buckbee Mears Co., St. Paul, MN), quartz windows on either side of the OTTLE, a gold minigrid counterelectrode, an aluminum bracket to hold the cell together, a gasket (General Electric RTV 112 Silicon rubber) between the front and back of the cell, two Hamilton valves (one for filling the cell, the other for connection of the reference electrode), and a compartment for the reference electrode. The reference electrode compartment was a glass tube (Kontes) with a female luer taper. The reference electrode was placed in the glass tube and sample solution added until the Vycor frit of the reference electrode was immersed into the sample solution. The reference electrode (Bioanalytical Systems, Lafayette, IN) was a Ag/AgCl type in 3.0 M NaCl (E_{ref} = −35 mV vs SCE). The Ag/AgCl reference electrode was compared to the laboratory standard SCE (a large-volume SCE constructed according to published procedures[37] and calibrated against a National Bureau of Standards electrode) before and after each titration, due to the possibility of contamination of the Vycor frit. The potential of the Ag/AgCl reference electrode varied by up to ±2 mV during its working lifetime. The gold minigrid working and counterelectrodes were connected on the outside of the gasket to pieces of aluminum foil, which were in turn used to complete the three-electrode electrochemical circuit.

Several spectroelectrochemical cells were also constructed in which the OTTLE was reticulated vitreous carbon, purchased from Teledyne Thilbrick (Dedham, MA) and cut into thin (0.08-mm) slices with a modified cheese slicer (wire and roller type). As in the gold minigrid cells, the reticulated vitreous carbon was connected to aluminum foil outside the rubber gasket.

Once the cells had been constructed, they were tested, first by spectroelectrochemical titration of methyl viologen and then cytochrome c according to liturature procedures.[38,39] Acceptable reproduction of literature data was obtained in each case (methyl viologen, $E^{\circ\prime}$ = −447 ± 1 mV, slope = 50.6 ± 0.5 mV as compared to −449 and 52 mV,[38] respectively; cytochrome c, $E^{\circ\prime}$ = 255.6 ± 1.8 mV, as compared to 255.0 mV,[39] slope = 58.4 mV). Following these tests of the spectroelectrochemical cells, titrations of cytochrome b_5 were begun. Relatively rapid electrochemical equilibration of the test solutions could be achieved only if three mediators were utilized. Thus, cytochrome b_5 titration solutions contained 0.2 mM $Ru(NH_3)_6Cl_3$, 0.2 mM $K_3Fe(CN)_6$, 1 mM methyl viologen, 0.100 M $NaClO_4$, 0.020 M phosphate buffer, pH 7.0, and 0.9–1.0 mg/mL cytochrome b_5.

The spectroelectrochemical cells were filled by first placing the solution in a degassing bulb equipped with a small magnetic stir bar, which was connected to the cell by a 180° Hamilton valve. The bulb was evacuated and then filled with argon while the solution was stirred. This procedure was repeated three times. Argon was then passed through the cell for several minutes with both Hamilton valves open. The degassing bulb and cell were then tipped to fill the cell under argon gas pressure. When the solution had entered the reference electrode reservoir, the 180° Hamilton valve was closed and the reference electrode was inserted into the reservoir. The cell was then ready for the titration. It was placed into the spectrophotometer and connected to the potentiostat. The first spectrum was taken before any potential was applied. It was then reduced at −400 mV vs Ag/AgCl for 20 min, and a spectrum was taken after this time period. The measuring solution was then oxidized at 0 mV vs Ag/AgCl for 20 min and again reduced for 20 min, followed by the taking of another spectrum. This spectrum usually was identical with that obtained after the first reduction. Spectra were then recorded at equilibrium at the following potentials (vs Ag/AgCl): −290, −260, −230, −200, −170, −155, 0, −185, −215, −245, −275, −305 mV. The titration was completed in 2 h. Following completion of each titration, the reference electrode was compared with the laboratory standard SCE. The temperature of the laboratory SCE, as well as the temperature of the spectrophotometer cell compartment, was measured. Corrections[40] were made for the temperature difference between the spectrophotometer cell compartment, the laboratory standard electrode, and the standard hydrogen electrode used in reporting the potentials in Table I.

All spectra were saved on disks and later plotted both as absorbance and as difference spectra (each titration point—fully oxidized spectrum). Nernst plots were made based on the absorbance at 424 nm.

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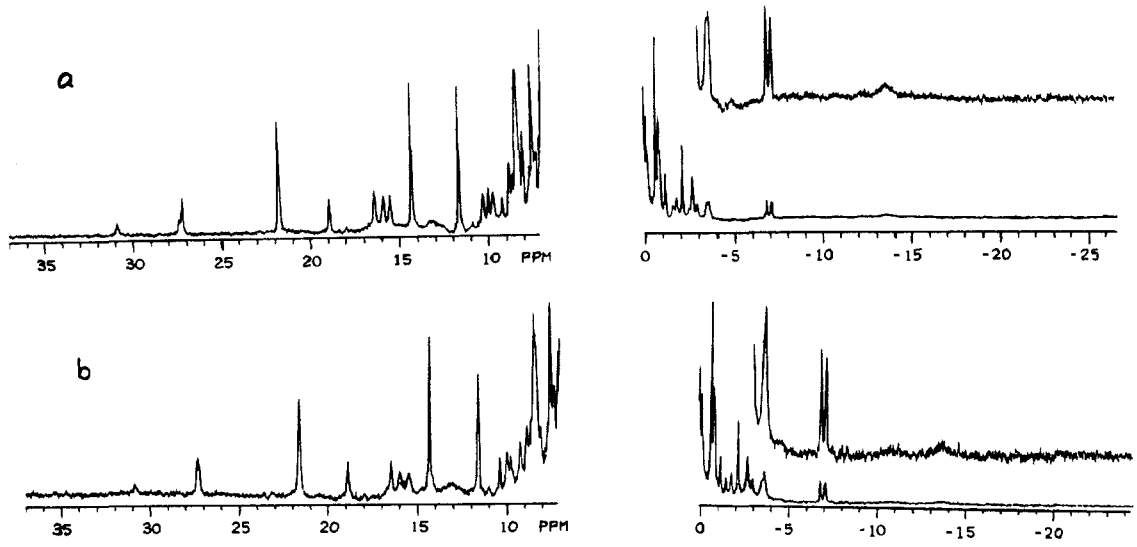

Figure 2. NMR spectra of cytochrome b_5 obtained from (a) bovine erythrocytes and (b) bovine liver microsomes after cleavage with trypsin. It should be noted that the relative intensities of the peaks at 22 ppm (5-CH_3, major) and 31 ppm (3-CH_3, minor) are the same for both samples. (The 1331 pulse sequence was not used in obtaining these spectra, and hence the methyl group peak intensities are reliable. The relative intensities of the 6- and 7-α-CH_2 peaks in the 18–20 ppm region are also the same for these two samples.) The pH of the erythrocyte b_5 was 6.9, while that of the microsomal protein was 7.2, each with no added buffer.

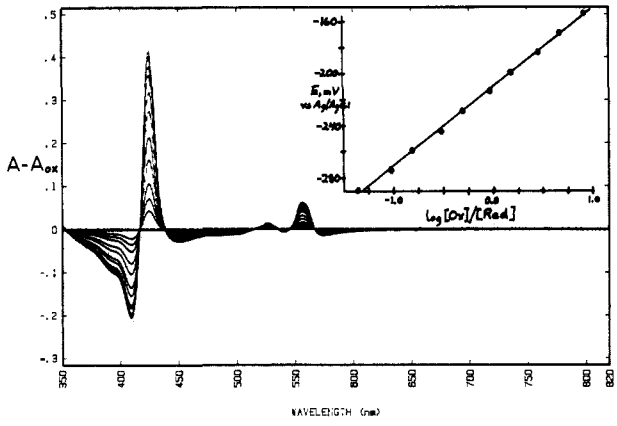

Figure 3. Difference spectra obtained from spectroelectrochemical titration of reconstituted cytochrome b_5 in the presence of the three mediators discussed in the text. The extremes represent the fully reduced minus fully oxidized spectra. Inset: Nernst plot of the same data. Slope = 61.4 mV, intercept = −8.7 mV vs SHE.

Results

NMR Spectroscopy. In Figure 2 parts a and b are shown the proton NMR spectra of erythrocyte and trypsin-cleaved microsomal cytochromes b_5, respectively, with emphasis on the hyperfine-shifted heme resonances. The ratio of major to minor heme orientation, calculated from the ratio of the intensities of the peaks at 22 ppm (5-CH_3, major) and 31 ppm (3-CH_3, minor) or of the peaks at 18.5 ppm (6-α-CH_2 minor) and 19.7 ppm (7-α-CH_2 major), is 8.9 for the microsomal protein and 9.1 for the erythrocyte protein, with an error of ±0.3 in the measurement. The chemical shifts of the heme resonances, previously assigned by other workers,[18,19] are the same for the two proteins. Hence, both the heme electron delocalization pattern and the ratio of major to minor heme orientations are the same for the cytochromes b_5 obtained from the two sources. Thus, electrochemical studies were performed utilizing trypsin-solubilized microsomal cytochrome b_5 because of the comparative ease in isolation of the protein. For simplicity, the trypsin-solubilized microsomal protein will hereafter be referred to simply as cytochrome b_5.

Electrochemistry. In Figure 3 is shown an example of the difference spectra obtained during electrochemical titration of native cytochrome b_5 utilizing the gold minigrid OTTLE described in the Experimental Section. Tight isosbestic points, as are evident in Figure 3, indicate that no denaturation of the protein occurred during the course of the titration. The data of Figure 3 were analyzed according to the Nernst equation (6), utilizing Beer's

$$E = E^\circ + (RT/nF) \ln [\text{ox}]/[\text{red}] \quad (6)$$

law to calculate the concentration ratio of oxidized to reduced protein for each of the difference spectra of Figure 3. The resulting Nernst plot, constructed from the data of Figure 3, is shown in the inset, where the slope should ideally be 58.9 mV at 24 °C; in our case, the slopes ranged from 59.0 to 61.4 mV (Table I). The intercept of the Nernst plot is the calculated reduction potential for cytochrome b_5 vs Ag/AgCl. The Ag/AgCl–SCE–SHE correction is then applied, utilizing the temperature dependence of the potentials.[40] The data obtained for six titrations of the native and seven titrations of the reconstituted protein are summarized in Table I, where it can be seen that the standard deviations in redox potential for the native and reconstituted proteins are ±1.6 and ±1.7 mV, respectively.

The solutions used for the last two titrations of the reconstituted protein listed in Table I were stored in their respective spec-

Table I. Reduction Potentials of Native and Reconstituted Cytochrome b_5 (μ = 0.120 M, pH 7.2, T = 24 ± 1 °C)

trial	$E^{\circ\prime}$, mV (vs NHE)	slope, mV	R^2
	Native Protein		
1	−0.4	61.1	0.9978
2	−0.2	58.7	0.9976
3	−3.8	59.5	0.9988
4	−0.7	59.0	0.9989
5	−3.1	60.0	0.9985
6	−3.3	59.7	0.9976
av	−1.9 ± 1.6		
	Reconstituted Protein		
1	−7.7	61.1	0.9995
2	−10.4	61.1	0.9987
3	−8.2	61.2	0.9983
4	−11.7	60.0	0.9994
5	−11.2	61.4	0.9982
6	−8.7	61.4	0.9986
7	−11.9	62.3	0.9996
av	−10.0 ± 1.7		
6[a]	−1.8	61.4	0.9996
7[a]	−1.8	59.8	0.9990

[a] Titrated again after 3 days' storage in an argon-filled desiccator. Only the oxidation cycle was used in calculating the redox potential because of oxygen leakage during the following reduction cycle.

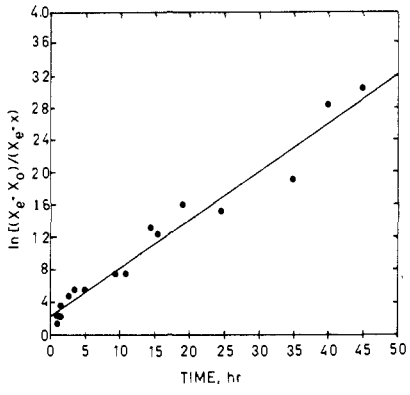

Figure 4. Kinetic data for the rate of heme rotation in H_2O, under the conditions used for electrochemical titrations. Slope = $k_1 + k_{-1}$ = 0.0594 h^{-1}, k_1 = 0.0535 h^{-1}, $t_{1/2}$ = 13.0 h.

Table II. Summary of NMR Kinetic Data for the Heme Reorientation of Ferricytochrome b_5 in the Presence of Electrochemical Mediators and Electrolyte

conditions[a]	$k_1 + k_{-1}$, h^{-1}	k_1, h^{-1}	$t_{1/2}$(minor), h	x at 65 min
H_2O, pH 7.0, 24 °C	0.0594	0.0535	13.0	0.61
H_2O, pH 7.0, 24 °C	0.0605	0.0544	12.7	0.56
H_2O, pH 7.0, 24 °C	0.0739	0.0665	10.4	0.64
av			12.0 ± 1.2	0.60 ± 0.03
D_2O, "pH" 7.0, 24 °C	0.0395	0.0356	19.5	
D_2O, "pH" 7.0, 24 °C	0.0444	0.0399	17.4	
D_2O, "pH" 7.0, 24 °C	0.0483	0.0435	16.0	
av			17.6 ± 1.4	

[a] Each solution contained 0.2 mM $Ru(NH_3)_6Cl_3$, 0.2 mM $K_3Fe(CN)_6$, 1 mM methyl viologen, 0.100 M $NaClO_4$, 0.020 M phosphate buffer, and 1 mM cytochrome b_5.

troelectrochemical cells in the oxidized form in an argon-flushed desiccator at room temperature for 3 days and then retitrated, giving, in each case, an $E^{\circ\prime}$ of −1.8 mV. Three days is about equal to 6 half-lives for heme rotation for ferricytochrome b_5 at pH 7.0 (vide infra), so the ratio of major to minor forms should have been within experimental error of the equilibrium ratio. Unfortunately, we were unable to verify this directly because the electrochemical solutions were too dilute (0.06–0.07 mM in cytochrome b_5) for NMR measurements.

In order to determine the rate of heme rotation in the electrochemical solutions, samples that contained the same concentrations of mediators, buffer, and electrolyte, but were 1 mM in reconstituted cytochrome b_5, were prepared for kinetic investigation by NMR spectroscopy. The samples were maintained at 25 °C and were integrated over the 18–20 ppm range where one of the diastereotopic protons of the 6-α-CH_2 of the minor and one of those of the 7-α-CH_2 of the major resonate. From the relative integrated intensities of these two signals the term ln $[(X_e - X_0)/(X_e - x)]$ could be evaluated and plotted as a function of time, as shown in Figure 4. Using the value $k_1/k_{-1} = 9$, a value of $k_1 = 5.81 \times 10^{-2}$ h^{-1} is obtained. This leads to a half-life for the minor to major transformation of 13 h for the data of Figure 4. Three experiments gave an average $t_{1/2}$ of 12.0 ± 1.2 h (Table II). Similar measurement of the rate of heme rotation in D_2O in the presence of the electrochemical mediators and electrolyte gave an average $t_{1/2}$ for minor to major conversion of 17.6 ± 1.4 h, somewhat shorter than the value obtained by LaMar and McLachlan in the absence of electrochemical mediators (21.5 h).[20] The fact that the rate of heme rotation is slower in D_2O than in H_2O implies that there must be rate-determining step(s) that involve hydrogen-bond breaking. Presumably, this hydrogen-bond breaking occurs by the flexing of the protein helices that make up the heme pocket.

In order to evaluate the reduction potentials of hypothetical cytochrome b_5 samples having pure major or pure minor heme orientation, the half-life must be correlated with the time course of the electrochemical titration. This correlation is made more complex by the fact that ferrocytochrome b_5 has a much slower (immeasurably slow) rate of heme reorientation than does ferricytochrome b_5.[43] Thus, it can be assumed that no heme rotation occurred during the time the protein was in the reduced form. The procedures used for preparing the reconstituted protein for the electrochemical titration were followed methodically each time, so that the time course of each titration was the same: Reconstitution of apoprotein, addition of electrolyte, pH adjustment, degassing, and loading the sample into the electrochemical cell required 15 min. The cytochrome b_5 sample was then reduced for 20 min, oxidized for 20 min, and then again reduced for 20 min. If one assumes that the protein was in the reduced form for two-thirds of this time, then during the first 75 min following reconstitution the protein was in the oxidized form for 35 min. At this point the potential step spectrophotometric titration was begun. This titration took 2 h to complete the cycle between oxidizing and reducing potentials. It is reasonable to assume that by the end of the titration the protein had spent half of the time in each oxidation state. Thus, the cytochrome b_5 was in the oxidized state for ~30 min by the midpoint of the titration, or a total of 65 min following reconstitution. This would mean that the reconstituted sample for which the reduction potential was measured to be −10.0 ± 1.7 mV was composed of 60% major and 40% minor heme orientation, based upon the average $t_{1/2}$ value of 12 h. By use of these values and the ratio at equilibrium, the reduction potentials of the pure heme orientations are major E° = +0.8 mV vs NHE, minor E° = −26.2 mV vs NHE, and ΔE° = 27.0 mV.

Discussion

The heme environment and major:minor ratio of erythrocyte cytochrome b_5 have been shown by NMR techniques to be indistinguishable from that of trypsin-cleaved microsomal cytochrome b_5 (Figure 1). This is not surprising, since the two proteins differ only in the amino and carboxyl terminal chain lengths, with the erythrocyte protein having 13 and 11 additional amino acids for bovine erythrocyte b_5 I and II, respectively; both termini of the protein are far distant from the heme pocket.[12]

Electrochemical titrations of cytochrome b_5 carried out in this study utilized the spectroelectrochemical technique first described by Hawkridge and Kuwana[33] and Heineman and co-workers[34] and first utilized for metalloproteins by Gray and co-workers for the blue copper proteins azurin, plastocyanin, and stellacyanin.[42] Attempts to increase the rate of electrochemical equilibration and thus shorten the titration time by means of electrode surface modification along the lines suggested by the work of Hill and co-workers[39,43] were unsuccessful. In particular, cyclic voltammetric measurements of cytochrome b_5 on two-dimensional gold discs in the presence of 4,4′-bipyridyl or 4-(1-methylpyridinium)-4′-pyridine[44] did not produce reversible waves. (We had thought that the latter reagent might compliment the charge type of cytochrome b_5 as the former is believed to do for cytochrome c.[42]) Reversible cyclic voltammetric waves were obtained on glassy carbon in the presence of magnesium ions, as suggested by the earlier work of Hill and co-workers on ferredoxin.[45] However, $^1/_2$ h of cycling the potential was required in order to produce reversible waves. Furthermore, we were unable to apply this system to the spectroelectrochemical technique because the reticulated vitreous carbon that we desired to use as the OTTLE had much larger holes than the gold minigrid and its thickness was approximately 16 times greater; thus, excessive time was required for diffusion of cytochrome b_5 to the electrode. Thus, we focused our attention on the gold minigrid OTTLE and accepted the three-mediator system typically used for bulk electrochemical titration of proteins[32,40] as the best for achieving relatively rapid electrochemical equilibration. A typical time scale

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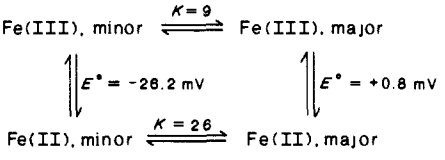
for the titrations, discussed above, meant that by the midpoint of the titration the reconstituted cytochrome b_5 had been in the oxidized form for ~65 min. NMR measurements of the ratio of major to minor heme orientations showed that after 65 min the ratio was typically 60:40.

The spectroelectrochemical titrations of native cytochrome b_5 done in this laboratory gave a slightly different redox potential than that reported by Reid and co-workers[46] under comparable ionic strength conditions: The $E^{\circ\prime}$ value extrapolated to $\mu = 0.13$ M, pH 7.0, is about +7 mV,[46] whereas we have obtained $E^{\circ\prime} = -1.9$ mV under the same ionic strength and pH conditions. This small but consistent difference may be due to the difference in the anion used for adjusting the ionic strength (ClO_4^- in our case, phosphate buffer in the earlier study)[46] or to the fact that Reid and co-workers used only one mediator, $Ru(NH_3)_6^{3+}$,[46] whereas we have used three. The use of an upper, central, and lower mediator [$Fe(CN)_6^{3-}$ (E° = 424 mV vs SHE),[38] $Ru(NH_3)_6^{3+}$ (E° = 51 mV vs SHE),[47] and methyl viologen (E° = −449 mV vs SHE)[48]] has been described for bulk electrochemical titrations of proteins for some years as a means of achieving electrochemical equilibrium more rapidly during such titrations.[32] This may seem surprising since in the potential range of interest in these titrations greater than 99% of the ferricyanide is in the reduced form and methyl viologen in the oxidized form. In our hands, spectroelectrochemical solutions also achieved electrochemical equilibrium more rapidly (5–10 min per titration point) when three mediators were utilized than when only $Ru(NH_3)_6^{3+}$ was used as mediator (30–45 min per titration point).[49] In addition, there was no evidence of protein denaturation or adsorption on the electrode when three mediators were utilized, whereas use of $Ru(NH_3)_6^{3+}$ alone led to some denaturation or adsorption of protein on the gold minigrid electrode, as evidenced by the fact that the absorbance of the fully reduced protein was consistently lower at the end of the titration than it was at the beginning, when only one mediator was utilized.[49] We were, in fact, not able to obtain reproducible titration data utilizing only $Ru(NH_3)_6^{3+}$ as mediator. The difference in potential may therefore represent some constant difference in working electrode surface preparation between the two laboratories. However, for the purposes of the present study, this difference in reported redox potential is unimportant, since our main purpose is to compare the redox potentials of native and reconstituted cytochrome b_5.

In separate NMR studies we found that $Ru(NH_3)_6^{3+}$ interacts with the exposed heme carboxylate of cytochrome b_5, causing shifts of the 6-α-CH_2 protons as a function of the $Ru(NH_3)_6^{3+}$ concentration.[31,50] However, at the ionic strengths and concentrations of cytochrome b_5 and $Ru(NH_3)_6^{3+}$ utilized in the spectroelectrochemical titrations, the amount of cytochrome b_5 complexed to $Ru(NH_3)_6^{3+}$ amounts to ca. 1% of that present.[50]

Our NMR measurements of the rate of heme rotation in hemin-reconstituted apocytochrome b_5 in D_2O under the conditions utilized for electrochemical titrations, in comparison to those in which no additives were present,[20] indicate that this rate is not greatly affected by the presence of redox mediators. However, the rate is very dependent on whether the solvent is H_2O or D_2O. This must be an important clue to the mechanism of heme rotation. However, the details of this mechanism have not yet been elucidated.

The electrochemical results on the native and reconstituted proteins (Table I), coupled with separate NMR quantitation of the ratio of major to minor heme forms at times important in the electrochemical titration process, provide proof that the reduction potentials of the major and minor forms are indeed different, with the minor form having the more negative reduction potential. Assuming that the ratio of major to minor forms at the midpoint of the titration was 60:40, as measured separately by NMR spectroscopy, the reduction potential of the pure major form is +0.8 mV and of the minor form is −26.2 mV. With these values and the equilibrium constant for minor–major interconversion for the oxidized form, the equilibrium constant for minor–major interconversion for the reduced form can be calculated from a Hess's law cycle:



The result is that for the reduced form, $K = 26$, reflecting a larger free energy difference in the two orientations for the reduced form than for the oxidized form. Thus, we see that in the absence of unsymmetrical d_π orbital occupation [Fe(II), low-spin d^6], the shape of the protein pocket favors the major orientation over the minor by a significant amount. However, upon oxidation to Fe(III), with its low-spin d^5 configuration, the unequal population of the d_π orbitals works to counteract the shape selection of the protein pocket, due to a more favorable electron configuration for the minor form.

The more negative reduction potential for the minor form means that the ΔG for reduction of the minor form is less favorable (less negative) than that for the major form. However, for the interaction with methemoglobin it is the reverse reaction that is important: That is, cytochrome b_5 is maintained in its reduced form by NADH-cytochrome b_5 reductase and is thus used to reduce methemoglobin, at which point it becomes oxidized. Thus, the oxidation potential is the more relevant quantity, and we see that the minor form has the more favorable free energy of oxidation. However, the free energy difference between pure major and minor forms, calculated on the basis of $\Delta E^{\circ\prime} = 27$ mV, is only ca. 620 cal/mol, probably not physiologically important. Furthermore, while the minor form is slightly better at reducing methemoglobin, it is by the same token slightly poorer at being rereduced by NADH-cytochrome b_5 reductase, so the effect should cancel out if both the oxidation and reduction steps involving cytochrome b_5 are rate determining, as has been suggested.[51] In addition, the analysis thus far assumes that the reduction of methemoglobin is controlled entirely by the thermodynamics of the system, whereas in reality, kinetics probably play a more important role. Nevertheless, it is interesting to see that there is a measurable difference in reduction potential between the major and minor forms. The origin of the difference (orientation of the histidine planes approximately over pyrrole rings I and III in the minor form and over II and IV in the major form, or subtle factors involving the interaction of the heme substituents with the protein side chains lining the heme pocket) has yet to be clearly defined. However, while the difference in reduction potential is relatively small and may not be physiologically important, the detailed correlation between structural features and reduction potential is very important for understanding the reduction potentials of heme proteins in general. Additional studies aimed at elucidating the separate effects of heme substituents and orientation of the histidine planes are in progress.

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Registry No. Cytochrome b_5, 9035-39-6; heme, 14875-96-8.

Communications to the Editor

The Reaction of Dimethylsilylene with Carbon Monoxide in Low-Temperature Matrices

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The gas-phase reaction of methylene with carbon monoxide to form ketene has been studied for both singlet and triplet methylene.[1] Calculated potential energy surfaces suggest that the barrier for addition of singlet methylene to carbon monoxide is low (less than 1 kcal/mol) or nonexistent.[2] We expect the corresponding reaction of dimethylsilylene to have a similar low barrier. In this communication we report evidence for a reaction of dimethylsilylene with carbon monoxide resulting in the formation of a species in which the CO is bonded to the silicon atom.

Dimethylsilylene, $(CH_3)_2Si$, has a singlet ground state with an empty p orbital and a lone pair in an sp^2 hybrid orbital. The 1-2 hydrogen migration which occurs so readily in the corresponding alkylcarbenes[3] has a calculated barrier of about 170 kJ/mol[4] and occurs in low-temperature matrices for dimethylsilylene only upon photoexcitation, so that this form of divalent silicon can be readily generated and trapped in low-temperature matrices.[5] Bimolecular reactions of dimethylsilylene with small reactant molecules can be conveniently followed in low-temperature matrices and can be used to produce reactive species which have structures not normally found in silicon-containing compounds.[6]

Most of the experiments were conducted in solid argon by using conventional low-temperature matrix techniques with an Air Products closed cycle helium refrigerator operated at temperatures between 15 and 40 K. Infrared spectra were recorded on a Perkin Elmer 983-G spectrophotometer and UV-vis spectra on a Perkin Elmer 552 spectrophotometer. Gas mixtures of CO in Ar were passed over crystalline dodecamethylcyclohexasilane, **1**, and deposited on a CsI window. Spectra were recorded before and after irradiation with either a low-pressure or medium-pressure mercury lamp. Additional experiments were carried out with **1** dissolved in 3-methylpentane glasses at 77 K in a quartz dewar. Carbon monoxide was obtained from Matheson, isotopically labeled CO from MSD Isotopes, and **1** and dimethyldiazidosilane, **2**, from Petrarch Chemicals.

Figure 1 displays infrared spectra of **1** in a matrix of Ar with 2% CO before and after irradiation. The intense product band appearing at 1962 cm^{-1} is assigned as the CO stretch of carbon monoxide that has formed an adduct with dimethylsilylene. This infrared band only appears when both **1** and CO are present and have been irradiated. The band increases following warming of the irradiated matrix to 40 K to allow migration of CO and to a lesser extent dimethylsilylene. In addition there is a change in the relative intensities of the overlapping features within the band presumably attributable to the annealing of different sites within the argon lattice and the possible influence of neighboring molecules on vibrational frequencies. The 1962-cm^{-1} band also appears when dimethyldiazidosilane, **2**, is irradiated in the presence of CO. Dimethylsilylene has been shown to be formed by photolysis of **2**.[7]

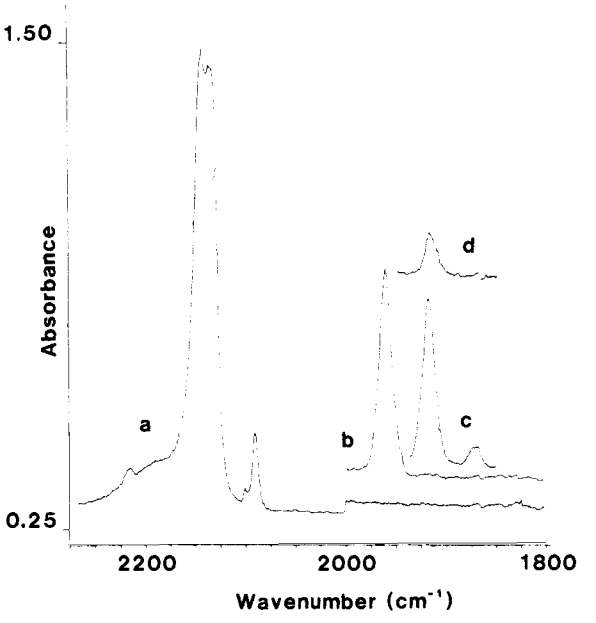

Figure 1. Infrared spectra of **1** in solid argon with 2% CO: (a) before irradiation, (b) after irradiation with $^{12}C^{16}O$, (c) after irradiation with $^{13}C^{16}O$, and (d) after irradiation with $^{12}C^{18}O$.

Table I. Experimental and Calculated Wavenumbers (cm^{-1}) for the CO Stretch

species	exptl wavenumber (isotope shift)	AM1[a] (shift)	MNDO[a] (shift)	3-21G[b]
$(CH_3)_2Si^{12}C^{16}O$	1962	2208	2310	2351
$(CH_3)_2Si^{13}C^{16}O$	1918	2156	2255	
	(44)	(52)	(55)	
$(CH_3)_2Si^{12}C^{18}O$	1915	2162	2262	
	(47)	(46)	(48)	
$^{12}C^{16}O$	2149[c]	2267	2382	2316

[a] Calculated wavenumber using AM1 or MNDO Hamiltonian in MOPAC4.[8a] [b] Calculated wavenumber using GAUSSIAN 86 with 3-21G basis set.[8b] [c] In argon matrix: Davies, J. B.; Hallam, H. E. *J. Chem. Soc., Faraday Trans. II* **1972**, *68*, 509.

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